Discrepancies in the German Pharmacopoeia procedure for quality control of Quillaja saponin extracts

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Abstract
This study focused on the evaluation of Quillaja saponin extracts with the additional quality designation DAB—which means the abbreviation of the German Pharmacopoeia (Deutsches Arzneibuch). This label suggests that Quillaja saponin extracts marked in this way are of pharmacopoeial quality and thus stand out from other Quillaja saponin extracts. The DAB ninth edition listed Quillaja saponin as a reagent. According to DAB, its quality must be checked by thin-layer chromatography (TLC), and three closely spaced zones in a defined retention factor (Rf) interval specify the saponin reagent. All the Quillaja saponin extracts obtained from different manufacturers and labeled as DAB quality complied with the TLC test. However, the analysis with high-performance liquid chromatography–quadrupole time-of-flight–mass spectrometry (HPLC–Q–ToF–MS) clearly showed additionally an intense peak pattern of Madhuca saponins in all measured samples. The TLC test for Mahua seed cake, which is the press residue from Madhuca longifolia, surprisingly showed the same three closely spaced zones in the defined Rf interval. The three zones could be identified as Mi-saponins from Madhuca after scraping and extracting them from the stationary phase of the TLC plate and subsequent measurement by HPLC–Q–ToF–MS. Therefore, the specification of the saponin reagent in DAB characterizes erroneously Madhuca saponins that are not listed as a saponin plant source for the saponin reagent.

KEYWORDS
adulteration, German Pharmacopoeia 9th ed., Madhuca longifolia, pharmacopoeial quality control, Quillaja saponin extract

1 | INTRODUCTION

Saponins from Quillaja saponaria Molina are used in various industrial sectors, such as in the feed industry as an additive to reduce the formation of the greenhouse gas methane and to reduce the production of ammonia.[1–3] Furthermore, saponins from Quillaja are used in the form of water-soluble extracts as an additive in the cosmetics industry and the food industry.[6,7] Saponins from Quillaja have undergone a transformation in the field of medicinal applications. Quillaja has traditionally been used as an expectorant. In recent years, the importance of highly purified Quillaja saponins as adjuvants in both veterinary and human vaccines has increased.[8–15] For example, Quillaja saponins are contained in a vaccine against Coronavirus disease.[16] Purity is basically a central criterion for Quillaja saponin extracts. Mixtures with inexpensive saponin sources are repeatedly described in the literature, for example, Quillaja saponins are blended with Yucca L. saponins[17] or as reported in

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a previous paper Madhuca J. F. Macbr. saponins from a press residue are detectable in commercially available Quillaja saponin extracts as an inexpensive saponin source.

The focus of this study is on Quillaja saponin extracts from the fine chemical trade that have the additional designation DAB/DAB 9, which is the abbreviation of the German Pharmacopoeia ninth edition. When DAB is used in the present text, the ninth edition is referred to. The Quillaja saponin extracts DAB analyzed within this study are different batches from different suppliers.

DAB suggests that the Quillaja saponin extracts labeled in this way are of pharmacopoeial quality. The abbreviation DAB means a distinguishing feature compared to other Quillaja saponin extracts offered in the fine chemicals trade. High product quality should be conveyed to the potential buyer.

In the DAB, there is no monograph on Quillaja saponaria, the parent plant of Quillaja saponin extracts. However, the DAB does contain a reagent called Saponin RN (reagent of national origin), listed in the Reagent List of the National Pharmacopoeia.

This Saponin RN is described as plant glycosides of Gypsophila L. species or Quillaja saponaria. The reagent is characterized as a white powder strongly irritating to sneeze with the indication of its solubility in water and organic solvents. According to DAB, the quality of this saponin reagent is verified by thin-layer chromatography (TLC). The saponin reagent is tested under the conditions and in the concentration as indicated under the test of the primrose root (monograph in DAB). A retention factor (Rf) value interval is specified for the saponin reagent within which the saponin zones must be located. The DAB describes in the general part—preceding the individual reagents—that the standards given for reagents are not necessarily sufficient for use as a drug or pharmaceutical excipient. In other words, Quillaja saponin extracts, according to DAB, is a reagent that would be more precisely and correctly described as "reagent according to DAB." Furthermore, a TLC quality control does no longer seem to be appropriate for such a complex product as Quillaja saponins. In addition, previous studies on Quillaja saponin extracts without the additional designation DAB have shown that they were partially falsified. This special constellation, which is encountered for these Quillaja saponin extracts with the additional designation DAB, suggests an investigation of the quality of commercially available Quillaja saponin extracts with the additional designation DAB based on the standard specified in the DAB by means of high-performance liquid chromatography–mass spectrometry (HPLC–MS).

2 | RESULTS AND DISCUSSION

According to DAB specifications for the saponin reagent, in daylight, three closely spaced brown to brownish zones with Rf values of about 0.1–0.3 should appear on the chromatogram sprayed with the

![Figure 1](image1.png)

**Figure 1** Thin-layer chromatograms (daylight) of a typical commercially available Quillaja saponin extract with the label DAB (Deutsches Arzneibuch) (left picture: immediately after spraying/drying; right picture: hours later with brownish zone color)

![Figure 2](image2.png)

**Figure 2** Total ion current chromatograms of commercially available Quillaja saponin extracts with the label DAB (Deutsches Arzneibuch) from four different suppliers (a–d). Mi-saponin B isomers (1, 2), Mi-saponin A (3), QS 18 (4), and QS 21 (5)
anisaldehyde reagent. This means that the quality of Quillaja saponin extracts designated DAB is considered confirmed if brown to brownish zones appear in the defined zone interval of Rf values 0.1–0.3 in the thin-layer chromatogram.

Figure 1 shows thin-layer chromatograms of typical commercially available Quillaja saponin extracts with the label DAB. All tested extracts show brownish zones in the range of the Rf value interval 0.1–0.3. The brownish zones have Rf values of 0.13, 0.17, and 0.21 that match the DAB specification.

The investigated Quillaja saponin extracts with the designation DAB correspond to the TLC test given in DAB, but high-performance liquid chromatography–quadrupole time-of-flight–mass spectrometry (HPLC–Q–ToF–MS) analysis shows for all extracts also the characteristic peak pattern of the Madhuca saponins. Chromatograms of Quillaja saponin extracts with the label DAB from four different suppliers are given in Figure 2. Extracted ion chromatograms are shown in the Supporting Information. Different batches were quite similar and show the same pattern of chromatographic peaks.

These saponin signals from Madhuca are relevant signals in the chromatogram for all extracts analyzed. The three largest saponin signals in all chromatograms are the Mi-saponins [M-H]- with theoretical m/z 1221.5910 (Mi-saponin A) and m/z 1353.6332 (two isomers of Mi-saponin B). In addition, Mi-saponin C and madhucoside
A and B with molecular masses $[M-H]^{-} \ m/z$ 1383.6438, 1485.6755, and 1515.6861 are present in the chromatograms. *Quillaja* saponins appear in the chromatogram with longer retention times compounds, such as QS 18 and QS 21, as doubly charged molecular ions $[M-2H]^{2-} \ m/z$ 1074.4812 and 993.4548. The mass error of all measured masses is less than 3 ppm, which is a reliable confirmation.

The German Pharmacopoeia ninth edition explicitly mentions only two plants used for the production of saponin extract: *Gypsophila* L. species or *Quillaja saponaria*. A s *Madhuca* is not listed as a source plant for saponin reagent in the DAB, the product does not comply with DAB.

Subsequently, Mahua seed cake, which is the press residue of the seed kernels of *Madhuca*, was tested by the DAB TLC method. Surprisingly, the Mahua seed cake shows the same three brownish zones in the TLC chromatogram as the previously tested *Quillaja* saponin extracts labeled DAB (see Figure 3).

Therefore, in the next step, after TLC of a *Quillaja* saponin extract with the label DAB, the chromatographic plate was cut in the middle of the separated zones and only one-half was treated with the spray reagent to make the three bands visible. Subsequently, the area at the level of the brown bands with the two larger Rf values was scraped off from the other half. This scraped-off stationary phase was mixed with 1 ml of an ethanol/water mixture and sonicated at 70°C for 15 min. After cooling and filtering through a membrane filter, this solution was measured by HPLC–Q-ToF–MS. In the HPLC chromatogram, the signals of Mi-saponin A and an isomer of

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**TABLE 1** Detected *Madhuca* and *Quillaja* saponins

<table>
<thead>
<tr>
<th>Peak</th>
<th>Peak labeling</th>
<th>Sum formula</th>
<th>Charge</th>
<th>Exact m/z</th>
<th>Accurate m/z</th>
<th>Error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>Mi-saponin B isomer 1</td>
<td>C63 H102 O31</td>
<td>−1</td>
<td>1353.6332</td>
<td>1353.6332</td>
<td>0.00</td>
</tr>
<tr>
<td>Peak 2</td>
<td>Mi-saponin B isomer 2</td>
<td>C63 H102 O31</td>
<td>−1</td>
<td>1353.6332</td>
<td>1353.6356</td>
<td>−1.77</td>
</tr>
<tr>
<td>Peak 3</td>
<td>Mi-saponin A</td>
<td>C58 H94 O27</td>
<td>−1</td>
<td>1221.5910</td>
<td>1221.5933</td>
<td>−1.88</td>
</tr>
<tr>
<td>Peak 4</td>
<td>QS 18</td>
<td>C98 H158 O51</td>
<td>−2</td>
<td>1074.4812</td>
<td>1074.4786</td>
<td>2.42</td>
</tr>
<tr>
<td>Peak 5</td>
<td>QS 21</td>
<td>C92 H148 O46</td>
<td>−2</td>
<td>993.4548</td>
<td>993.4530</td>
<td>1.81</td>
</tr>
</tbody>
</table>
Mi-saponin B are detected (see Figure 4). The corresponding mass spectra of the Mi-saponins and the *Quillaja* saponins QS 18 and QS 21 are depicted in Figure 5. Besides the molecular ion [M–H]⁻, all mass spectra of the Mi-saponins show chloride adducts [M+Cl]⁺. The mass spectra of QS 18 and QS 21 show doubly charged species of the molecular ion [M–2H]²⁻ and the chloride adducts [M–H+Cl]²⁻. Table 1 compares the exact m/z with the measured m/z of the peaks identified and shown in the chromatograms.

In a further step, the area of the unsprayed half of the thin-layer plate was scraped off at the level of the brown bands with the lowest Rf value. After analogous treatment as described above, the HPLC-Q-ToF–MS analysis showed another isomer of Mi-saponin B in the chromatogram. And thus, the three brown zones of the pharmacopoeia standard for the Saponin reagent RN are the Mi-saponins. In other words, the pharmacopoeial standard for Saponin reagent RN does not characterize *Quillaja* but *Madhuca* saponins.

Mahua seed cake is an inexpensive source of saponins, namely a press residue of *Madhuca* seed kernels, which may have been mixed with *Quillaja* saponins and processed into extracts for decades and in this way unintentionally and erroneously found its way into the pharmacopoeia as a quality characteristic.

### 3 | CONCLUSION

*Quillaja* saponin extracts with the additional designation DAB should convey to the potential buyer a high product quality. DAB is a distinguishing feature that characterizes the extracts from other *Quillaja* saponin extracts. However, the criterion DAB does not describe the quality of an extract listed in a pharmacopoeial monograph, but only that of a reagent.

Furthermore, the label DAB occurs in an older edition of the pharmacopoeia, which clearly indicates that even compliance with the individual standards for reagents is not necessarily sufficient for them to be used as medicinal products or pharmaceutical excipients.

In addition, the *Quillaja* saponin extracts analyzed with this designation are adulterated with *Madhuca* saponins. This means that in all those cases where *Quillaja* saponins have been used with the aim of exploiting their specific properties, the results, effects, and qualities are possibly falsely attributed to the presence of solely *Quillaja* saponins.

The most surprising finding is that the pharmacopoeia referred to in these *Quillaja* saponin extracts erroneously characterizes *Madhuca* saponins in the given test. It stands to reason that those adulterants containing *Madhuca* saponins have been in use for decades.

### 4 | EXPERIMENTAL

#### 4.1 | Chemicals, plant materials, and *Quillaja* extracts

18 Ω cm water was generated from a Milli-Q water purification system (Millipore). Acetonitrile, methanol, ethanol, and 1-butanol analytical grade were obtained from VWR (Fontenay-sous-Bois), glacial acetic acid from VWR (Fontenay-sous-Bois), sulfuric acid and anisaldehyde from Merck, and formic acid from Sigma-Aldrich. Samples of commercially available *Quillaja* saponin extracts received from four different suppliers were analyzed, whereby also different batches of the suppliers were included. Information about the origin of the adulterated samples is intentionally not given to avoid any possible conflicts. Mahua seed cake, which is the press residue from *Madhuca longifolia*, was obtained from Kaushal Herbs and Beej Bhandar. The anisaldehyde spray reagent for TLC was prepared by mixing 85 ml methanol, 10 ml glacial acetic acid, 5 ml sulfuric acid, and 0.5 ml anisaldehyde.

#### 4.2 | TLC

DAB allows ready-to-use plates for the TLC test of the Saponin RN. Silica gel GF 254 plates are prescribed in the DAB as the stationary phase. TLC Silica Gel 60 F254 from Merck was used. The mobile phase was the upper phase of a mixture of 10 parts by volume acetic acid, 40 parts by volume water, and 50 parts by volume 1-butanol. Further, according to DAB, the chromatography chamber was provided with enough of the mobile phase to obtain a layer of 5–10 mm height. The chamber was lined with filter paper, which was moistened with the mobile phase. The sealed chamber was left for 1 h at 20–25°C and conditioned. Ten milligrams of each *Quillaja* saponin extract was dissolved in 1.0 ml of 70% ethanol. Separately, 40 µl of each individual *Quillaja* saponin extract solution was applied as a line in such a way that band-like zones of 20 × 3 mm were created approximately 20 mm from the bottom edge and at least 20 mm from the side edges of a plate. Spots of different samples were applied at least 15 mm apart, and on a line parallel to the bottom edge of the plate following the DAB.

After evaporation of the solvent of the applied solutions, the plate was placed vertically in the chromatography chamber, keeping the starting points above the level of the mobile phase. The chamber was then closed and kept at a temperature of 20–25°C. After a run length of 12 cm had been reached, the plate was removed, dried at 105°C, then sprayed with approximately 10 ml of anisaldehyde reagent (for a 200 × 200 mm plate), and heated to 105°C for about 5 min and then evaluated in daylight.

#### 4.3 | HPLC

An Agilent Series 1260 HPLC system was used. The HPLC system was equipped with a quaternary pump, a vacuum degasser, an autosampler, and a UV-vis diode array detector (all modules from Agilent). HPLC was hyphenated with an Agilent 6520 Q-ToF.

For the analysis of *Quillaja* saponin extracts, a Poroshell 120 EC-C18 Eclipse column (50 × 3 mm ID; 2.7 µm particle size) from Agilent was used. The injection volume was a 5 µl aliquot of the sample. The mobile phase consisted of acetonitrile (solvent A), water (solvent B), and aqueous 1% formic acid (solvent C). The mobile phase
gradient was programmed as follows: Initial conditions: solvent A 9%, solvent B 81%, and solvent C 10%; 2.0 min: solvent A 13%, solvent B 77%, and solvent C 10%; 12.0 min: solvent A 25%, solvent B 65%, and solvent C 10%; 34.5 min: solvent A 40%; solvent B 50%, and solvent C 10%; 42.0 min: solvent A 60%; solvent B 30%, and solvent C 10%; 47.0 min: solvent A 100%; 54.0 min: solvent A 100%; 54.1 min: solvent A 9%; solvent B 81%, and solvent C 10%. The run time was 60 min. The flow rate was 0.7 ml/min.

Sample preparation for HPLC-Q-ToF-MS analysis: 50–100 mg of the individual Quillaja saponin extracts quality label DAB were dissolved in 10 ml of 18 MΩ cm water. Solutions were filtered through a membrane filter (0.45 µm). One hundred milligrams of Mahua seed cake was suspended with 10 ml of 18 MΩ cm water. Solutions were filtered through a membrane filter (0.45 µm). One hundred milligrams of Mahua seed cake was suspended with 10 ml of 18 MΩ cm water and extracted at 70°C for 1 h in an ultrasonic bath. After cooling to room temperature, the opaque suspension was filtered through a membrane filter (0.45 µm).

4.4 | MS

An Agilent 6520 quadrupole/time-of-flight instrument was used. Ionization was done by electrospray in the negative ion mode. The following ion source conditions were employed: drying gas temperature 350°C, capillary voltage 3750 V, nebulizer pressure 45 psi, fragmentor voltage 200 V, and drying gas flow 10.5 l/min.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests.

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REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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